THE RECOMBINEERING COMPANY
GENE BRIDGES

Technical Protocol

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Quick & Easy Conditional Knockout Kit (FRT/FLPe)

By Red®/ET® Recombination

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Please read

The products listed in this manual are for research purposes only. They are not designed for diagnostic or therapeutic use in humans, animals or plants. Success depends on following the protocols exactly as they are described. Do read the trouble-shooting guide before beginning your experiments. Red/ET Recombination is the intellectual property of Gene Bridges GmbH.

Safety

Some chemical reagents used with this system are dangerous if handled carelessly. Take care when using chemical reagents (such as isopropanol and ethidium bromide) and electrical apparatus (high-voltage power supplies, gel electrophoresis and electroporation apparatus). Follow the manufacturer's safety recommendations.

1 Conditional Knockout Kit (FRT/FLP)

Introduction

The ability to introduce virtually any mutation into the genome followed by gene targeting in embryonic stem (ES) cells provides a powerful approach for elucidating gene function in the whole animal. In many cases, however, the complete deficiency of a gene leads to embryonic lethality, precluding the analysis of gene function in later developmental stages or in the adult. This problem can be overcome by creating conditional knockout animals allowing a gene to be inactivated in a tissue- or temporal-specific fashion.
Typically, a conditional knockout allele is made by inserting loxP or FRT sites into two introns of a gene. Expression of Cre or FLP recombinase in the animal carrying the conditional knockout allele catalyzes recombination between the loxP and the FRT sites, respectively, and inactivates the gene. By expressing Cre or FLP recombinase from a tissue-specific promoter, the gene can be inactivated in a tissue-specific fashion.
A major limitation for generating conditional knockout animals is the difficulty and time it takes to make the appropriate targeting vector. The conventional approach is to find appropriate restriction enzyme sites that are located in or near the gene. These sites are then used to ligate together loxP or FRT sites and various other DNA fragments such as homology arms and a positive selection marker such as PGKneo. The problem with this approach is that restriction sites are often not available or inconveniently located thus severely limiting where loxP or FRT sites can be placed.

Red/ET Recombination makes it possible to introduce loxP or FRT sites and selectable markers anywhere in a gene, and greatly reduces the amount of time it takes to make a targeting vector.
Red/ET Recombination relies on homologous recombination *in vivo* in *E.coli* and allows a wide range of modifications with DNA molecules of any size and at any chosen position. Homologous recombination is the exchange of genetic material between two DNA molecules in a precise, specific and accurate manner. These qualities are optimal for engineering a DNA molecule regardless of its size. Homologous recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine. Because the sequence of the homology regions can be chosen freely, any position on a target molecule can be specifically altered. Red/ET recombination allows you to choose **homology arms as short as 50 bp** for homologous recombination, which can easily be added to a functional cassette by long PCR primers.
Zhang and coworkers demonstrated in 1998 for the first time that a pair of phage coded proteins (RecE and RecT) only need 42bp long homology arms to mediate the homologous recombination between a linear DNA molecule (e.g. a PCR product) and circular DNA (plasmid, BAC or *E. coli* chromosome). This method was used to disrupt the endogenous *lac*Z gene of *E. coli* strain JC9604 (Zhang et al 1998). One year later the system was extended by the same group in replacing *rec*E and *rec*T by their respective functional counterparts of phage lambda *red*α and *red*β (Muyrers et al. 1999).

The recombination process is strictly controlled since the necessary genes are located on an expression plasmid which carries a temperature-sensitive origin of replication and can therefore only be propagated at 30°C. Increasing the temperature to 37°C for a period of time results in a loss of the expression plasmid after recombination. In addition the expression of the proteins is tightly controlled by an inducible promoter opening just a short time window for the recombination process.

The Quick and Easy Conditional Knockout Kits from Gene Bridges, comprising a FRT/FLPe and a loxP/Cre Kit, are specifically designed to integrate FRT or loxP sites in large vector plasmids at any intended position without the need to use restriction enzymes within 2 weeks. Conditional targeting constructs can be generated either by a repetitive insertion of the functional cassette supplied with the kit (FRT-PGK-gb2-neo-FRT or loxP-PGK-gb2-neo-loxP) or by insertion of any other functional cassette offered by Gene Bridges (e.g. FRT-PGK-gb2-neo-FRT-loxP).

The "FRT-PGK-gb2-neo-FRT" cassette supplied with the kit is designed to allow kanamycin/neomycin selection in prokaryotic and eukaryotic cells, respectively. It combines a prokaryotic promoter (gb2) for expression of kanamycin resistance in *E.coli* with a eukaryotic promoter (PGK) for expression of neomycin resistance in mammalian cells. The prokaryotic promoter gb2 is a slightly modified version of the Em7 promoter; it mediates higher transcription efficiency than the generally used Tn5 promoter. The promoter of the mouse Phospho-glucokinase gene (PGK) is used as the eukaryotic promoter. A synthetic polyadenylation signal terminates kanamycin/neomycin expression. The cassette is flanked by FRT sites for later excision by Flp-recombinase.

The Flp integrase supplied with the kit was originally isolated from *Saccharomyces cerevisiae* where it mediates recombination between FRT (FLP Recombination Target) sites within yeast plasmids (Kilby *et al*. 1993). FRT sites are 34 bp DNA sequences comprised two 13 bp palindromes separated by an asymmetric 8 bp core. The integrase is a recombinase, which catalyzes DNA strand exchange between two aligned recombination sites, resulting in deletion, duplication, integration, inversion or translocation of sequences, according to the orientation of the recombination sites and the number of molecules involved. The only requirements for DNA rearrangement are the enzyme and the recombination sites, no additional cellular factors are necessary.
An improved FLP recombinase, called FLPe, was developed by cycling mutagenesis (Buchholz, Angrand and Stewart 1998). FLPe shows a four to tenfold improvement in recombinational activity compared to the "wildtype" FLP enzyme at temperatures between 37°C (optimal growth temperature for *E.coli*) and 40°C (mouse body temperature).

Deleter mice harboring FLPe achieve maximum target gene excision in both somatic and germ cells, demonstrating that FLPe is highly efficient in mice. It presents an important alternative to and a complement to the Cre-loxP system for *in vivo* genetic engineering. (Rodriguez *et al.* 2000)

Contents of the kit:

1. pRedET (tet): Red/ET expression plasmid (20 ng/µl, 20 µl)
2. FRT-PGK-gb2-neo-FRT template DNA: PCR-template (plasmid DNA) for generating a FRT flanked PGK-gb2-neo cassette (50 ng/µl, 20 µl)
3. FRT-PGK-gb2-neo-FRT PCR-product: PGK-gb2-neo cassette flanked by FRT sites and 50 bp long homology arms for the control experiment (100 ng/µl, 10 µl)
4. pSub-Hoxa11 + pRedET (tet): Glycerol stock of *E.coli* strain DH10B harboring the expression plasmid pRedET (tet) as well as a high copy plasmid containing 15 kb of the mouse Hoxa11 gene for the control experiment (500 µl, 25% glycerol)
5. pCI-FLPe: expression plasmid for enhanced FLP recombinase (20 ng/µl, 20 µl)
6. pSub-Hoxa11-FRTneo: Glycerol stock of *E.coli* strain HS996 harboring a high copy plasmid containing 15 kb of the mouse Hoxa11 gene and a FRT flanked cassette inserted into the second intron of the Hoxa11 gene (control experiment; 500 µl, 25% glycerol)
7. pSub-Hoxa11-FRT: Glycerol stock of *E.coli* strain HS996 harboring a high copy plasmid containing 15 kb of the mouse Hoxa11 gene and a single FRT site inserted into the second intron of the Hoxa11 gene (control experiment; 500 µl, 25% glycerol)
8. This manual with protocols, maps and sequences

Please store tubes 1-3 and 5 at -20°C, store tubes 4, 6 and 7 at -80°C.

Please note: All materials necessary for the control experiment are provided with this kit. You must order your oligonucleotides (PCR primer) according to your experimental design before starting. High quality oligos yield highest recombination efficiencies.

2 Experimental Outline

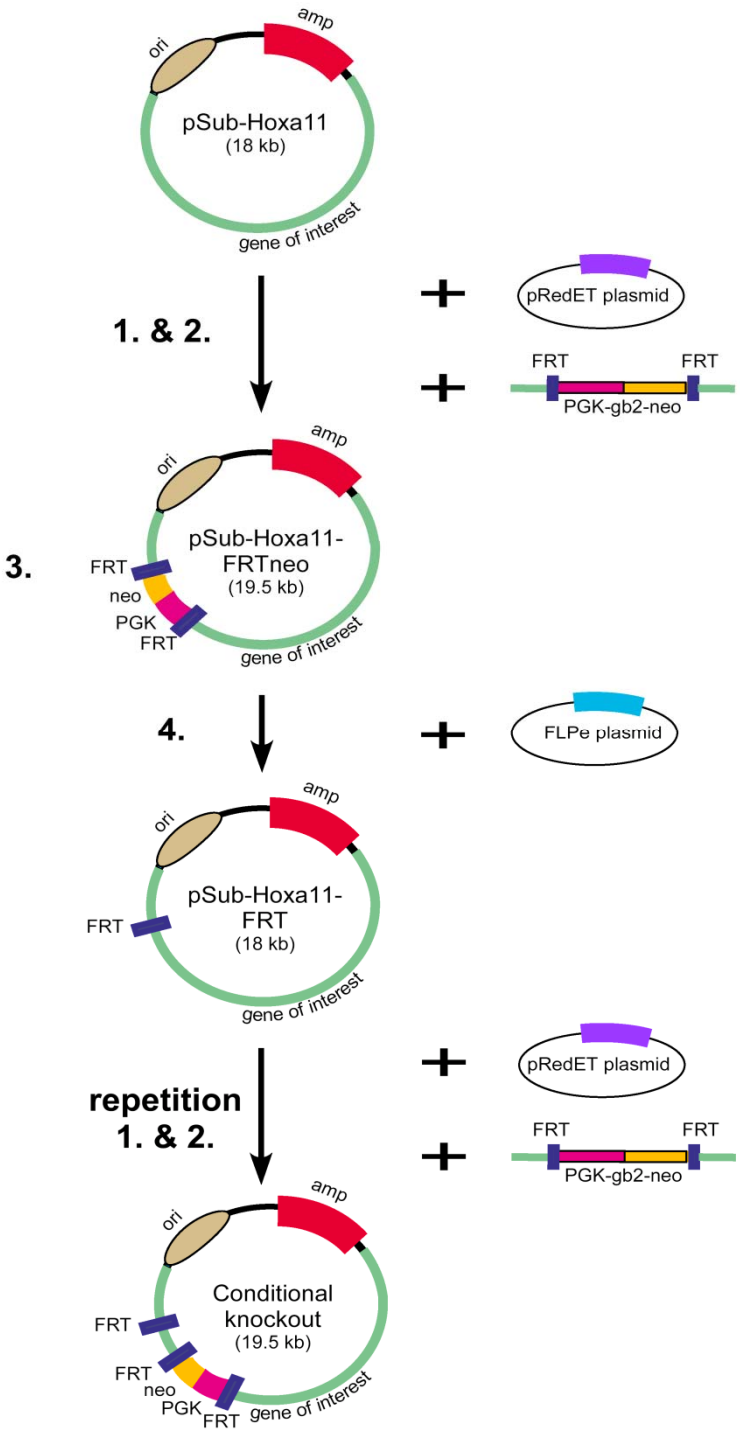


Figure 1: Flowchart shows the experimental outline for the generation of a conditional knockout construct based on FRT sites.

Figure 1:

1. **Transform *E. coli* cells harboring your plasmid with the expression plasmid pRedET (Figure 7, tube 1).** For your convenience this step has already been performed for the control experiment (control experiment tube 4). Prepare your PCR product using the 'FRT-PGK-gb2-neo-FRT template DNA' (Figure 9, tube 2) as template. Plate and grow **at 30°C.**

2. **Red/ET Recombination step**. The expression of genes mediating Red/ET is induced by the addition of L-arabinose and a temperature shift from 30°C to 37°C. After induction, the cells are prepared for electroporation and the PCR product (control experiment: tube 3 'FRT-PGK-gb2-neo-FRT PCR product'), which includes the homology arms, is electroporated.

3. **Selection for colonies carrying the modified plasmid.** Only colonies carrying successfully modified plasmids will survive kanamycin selection on the agar plates. Subsequent DNA mini preparation and check PCR are used to confirm the successful integration of the functional cassette. In most cases an additional re-transformation step is required to separate the modified plasmid from all copies of the original plasmid.

4. **FLPe Recombination step**. The FLPe expression plasmid pCI-FLPe (Figure 8, tube 5) is transformed into the cells harboring the plasmid with the inserted FRT-PGK-gb2-neo-FRT cassette (control experiment tube 6). Plate and grow **at 30°C.** Expression of FLPe recombinase is induced by a temperature shift to **37°C.** DNA mini preparation and check PCR are used to confirm the successful recombination step. An additional re-transformation step is required to separate the modified plasmid from all copies of the original plasmid. Take 'pSub-Hoxa11-FRT (tube 7) as control for the final product of the control reaction.

   Repetition of steps 1 & 2 can be performed to integrate the FRT-PGK-gb2-neo-FRT cassette at a second location (e.g. into another intron). The result will be a FRT-based conditional targeting construct.

3 How Red/ET Recombination works

In Red/ET Recombination, also referred to as λ -mediated recombination, target DNA molecules are precisely altered by homologous recombination in *E.coli* which express the phage-derived protein pairs, either RecE/RecT from the Rac prophage, or Redα/Redβ from λ phage. These protein pairs are functionally and operationally equivalent. RecE and Redα are 5‘- 3‘ exonucleases, and RecT and Redβ are DNA annealing proteins. A functional interaction between RecE and RecT, or between Redα and Redβ is also required in order to catalyze the homologous recombination reaction. Recombination occurs through homology regions, which are stretches of DNA shared by the two molecules that recombine (Figure 2). The recombination is further assisted by λ-encoded Gam protein, which inhibits the RecBCD exonuclease activity of *E.coli*.

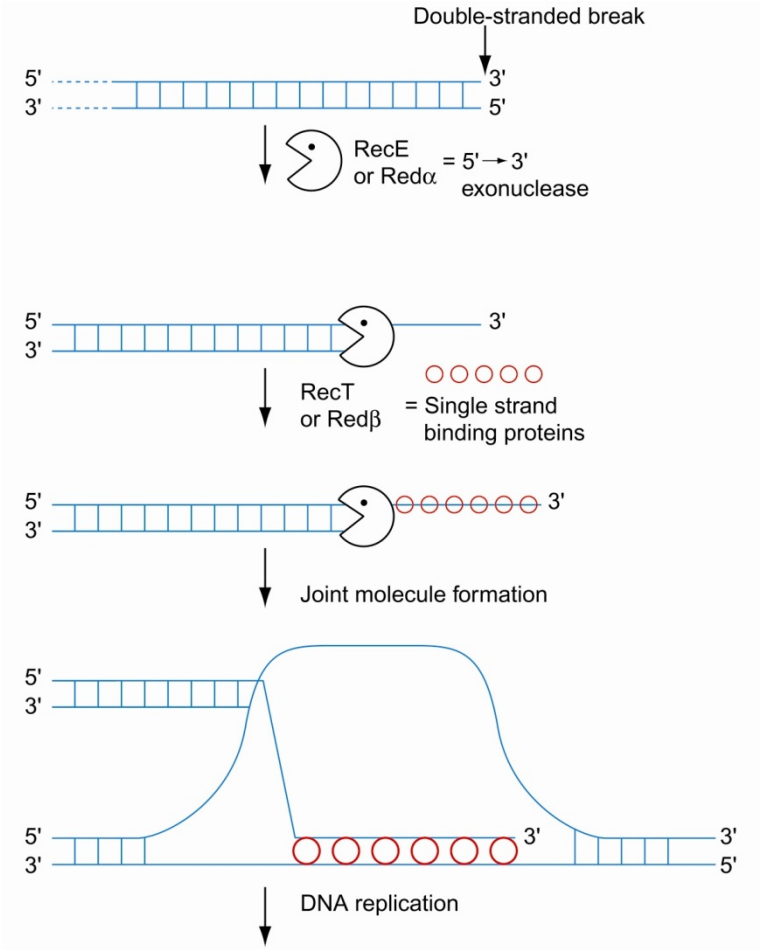


Figure 2: Mechanism of Red/ET Recombination.

Double-stranded break repair (DSBR) is initiated by the recombinase protein pairs, RecE/RecT or Redα/Redβ.

First Redα (or RecE) digests one strand of the DNA from the DSB, leaving the other strand as a 3' ended, single-stranded DNA overhang. Then Redβ (or RecT) binds and coats the single strand. The protein-nucleic acid filament aligns with homologous DNA. Once aligned, the 3' end becomes a primer for DNA replication.

The λ recombination proteins can be expressed from a plasmid (Figure 5) and are therefore transferable to any *E. coli* strain.

pRedET (Figure 7) carries the λ phage *red*γβα operon expressed under the control of the arabinose-inducible pBAD promoter (Guzman *et al.* 1995) and confers tetracycline resistance.

The pBAD promoter is both positively and negatively regulated by the product of the *ara*C gene (Schleif, 1992). AraC is a transcriptional regulator that forms a complex with L-arabinose. Arabinose binds to AraC and allows transcription to begin. In the presence of glucose or the absence of arabinose, transcription is blocked by the AraC dimer.

The plasmid carries the *red*α, β, γ genes of the λ phage together with the *recA* gene in a polycistronic operon under the control of an inducible promoter. The recombination window is therefore limited by the transient expression of Red proteins. Thus, the risk of unwanted intra-molecular rearrangement is minimized.

While constitutive expression of the *red* γ gene has a toxic effect in DH10B (recA-) cells under some conditions, thus limiting the efficiency of recombination, tightly regulated expression of the γ gene together with simultaneous expression of the *red*α and β genes allows efficient homologous recombination between linear DNA fragments and plasmids resident in cells such as DH10B.

pRedET is a derivative of a thermo-sensitive pSC101 replicon, which is a low copy number plasmid depending on the oriR101. The RepA protein encoded by plasmid pSC101 is required for plasmid DNA replication and the partitioning of plasmids to daughter cells at division (Miller, Ingmer and Cohen 1995). Because the RepA protein is temperature-sensitive (Ts), cells have to be cultured at 30°C to maintain the plasmid. pSC101 derivatives are easily curable at 37°C to 43°C.

Experiments have shown that the copy number of the plasmid decreases by about 80% during four generations of bacterial cell growth at 42°C. After return of the cultures to 30°C, approximately the same number of generations of bacterial cell growth is required for the copy number of the plasmid to return to the level observed before (Miller, Ingmer and Cohen, 1995).

Since the plasmid is based on oriR101 it can be propagated in *E.coli* together with most ColE1-derived plasmids.

4 Oligonucleotide Design for Red/ET Recombination

To target your plasmid at the site(s) of choice, you will need to attach short homology regions to the functional cassette carrying the selectable marker. This is most conveniently done by ordering two oligonucleotides for use in PCR amplification (Figure 3). Each oligonucleotide consists of two (or, if desired, three) parts:

1. Required Part A (A' for the second oligonucleotide) is the homology region shared by the target molecule and the linear molecule. The homology regions are the 50 bp directly adjacent to either side of the insertion site. The exact sequences of the homology regions can be chosen freely, depending on the position on the target molecule to be modified.

2. Optional Part B (B´ for the second oligonucleotide): This part of the oligonucleotide allows the incorporation of useful sequences, such as restriction sites. If the introduction of such operational sequences is not needed, this part can simply be omitted from the oligonucleotide design.

3. Required Part C (C' for the second oligonucleotide): This sequence, usually 18 to 24 nucleotides long, primes the PCR amplification of the selectable marker from the provided template.

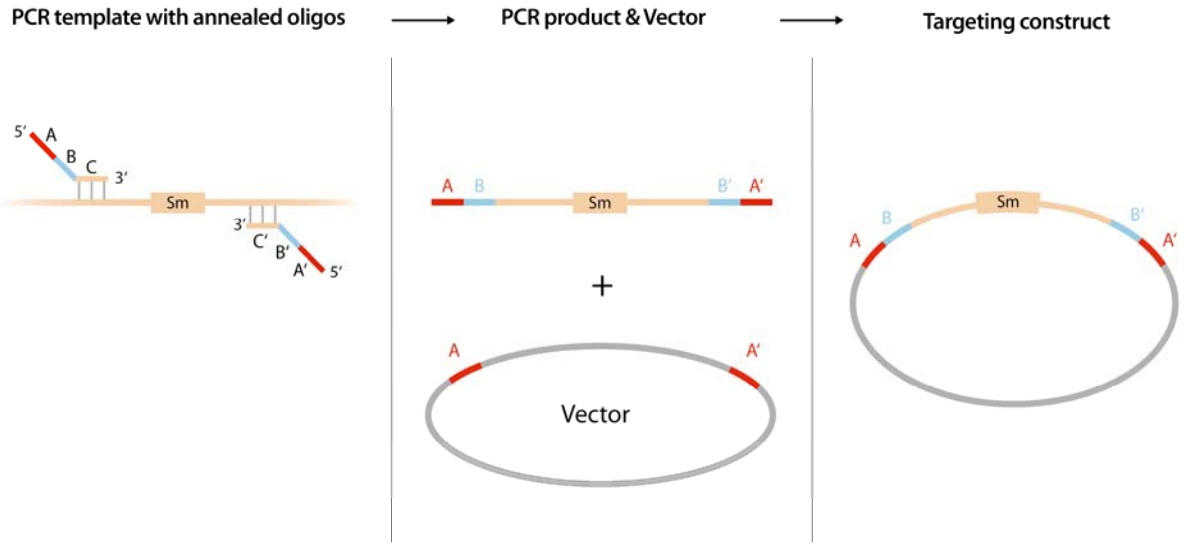


Figure 3: Practical steps involved to insert a fragment by Red/ET recombination.

The two oligos below were used to add the 50 bp homology regions (*italics*) for Red/ET Recombination to the FRT-PGK-gb2-neo-FRT cassette (Figure 9) used in the control reaction. The parts of the oligos which serve as PCR primers for amplification of the cassette are underlined. These two oligos are not supplied with the kit, but the resulting PCR product is supplied (tube 3).

Oligo 1:

5'-*TGATCAGAAGTCAGGCTGACAAAGACCCCTCAGCCGCCCCAGATGTTAAG*AA TTAACCCTCACTAAAGGGCG-3

Oligo 2:

5'-*CATGCATCCTGGCCCCAGGCTTTCCTGCTTGCCGCCATGATTTAGCCCTC*TA ATACGACTCACTATAGGGCTC-3'

Oligonucleotide Design for your target sequence:

I) Choose 50 nucleotides $(N)_{50}$ directly adjacent upstream (5') to the intended insertion site. Order an oligonucleotide with this sequence at the 5' end. At the 3' end of this oligo include the PCR primer sequence for amplification of the FRT-PGK-gb2-neo-FRT cassette, given in *italics* below.

Upper oligonucleotide (oligo 1): 5'-$(N)_{50}$ **AATTAACCCTCACTAAAGGGCG* -3'

II) Choose 50 nucleotides $(N)_{50}$ directly adjacent downstream (3') to the intended insertion site and transfer them into the ***reverse complement orientation***. Order an oligonucleotide with this sequence at the 5' end. At the 3' end of this oligo, include the 3' PCR primer sequence (also in reverse complement orientation) for the FRT-PGK-gb2-neo-FRT cassette, given in *italics* below.

Lower oligonucleotide (oligo 2): 5'-$(N)_{50}$ **TAATACGACTCACTATAGGGCTC* -3'

If desired, include restriction sites or other short sequences in the ordered oligo(s) between the 5' homology regions and the 3' PCR primer sequences (*).

5 Media for Antibiotic Selection

All antibiotics are available from Sigma. Stock solutions should be stored at -20ºC. For selective LB medium, the antibiotic is dissolved in LB medium to the indicated working concentration:

1. Chloramphenicol stock solution c = 30 mg/ml dissolved in ethanol. Working concentration 15 μg/ml for BACs/low-copy plasmids and 50 μg/ml for high-copy plasmids.

2. Ampicillin stock solution c = 100 mg/ml dissolved in 50% ethanol. Working concentration 50 μg/ml for BACs/low-copy plasmids and 100μg/ml for high-copy plasmids.

3. Tetracycline stock solution c = 10 mg/ml dissolved in 75% ethanol. Working concentration for pRedET 3 μg/ml, for high-copy plasmids 10 μg/ml. Tetracycline is light sensitive.

4. Kanamycin stock solution c = 30 mg/ml dissolved in ddH_20. Working concentration 15 μg/ml for BACs/low copy plasmids and 50 μg/ml for high-copy plasmids.

5. Hygromycin stock solution c = 50 mg/ml dissolved in ddH_20. Working concentration 20 μg/ml for BACs/low copy plasmids and 50 μg/ml for high-copy plasmids.

Selective LB plates are made by adding 15 g agar to 1 L LB medium. After boiling, cool to approx. 50°C, add the required antibiotics to yield the appropriate working concentrations and pour into petri dishes.

L-arabinose stock solution

Use 10% **L-arabinose** (Sigma A-3256) in ddH_2O, fresh or frozen in small aliquots at -20°C. Use 50 µl stock solution per 1.4 ml LB for induction of recombination protein expression from pRedET. Frozen aliquots should not undergo more than three freeze-thaw cycles.

6 Technical protocol

6.1 Generation of a functional cassette flanked by homology arms

PCR

The oligonucleotides are suspended in ddH_2O at a final concentration of 10 µM. We present as an example a standard PCR protocol for the use of Phusion DNA Polymerase (Finnzyme). However, any other DNA Polymerase together with the corresponding PCR protocol according to the instructions of the manufacturer should yield satisfactory results.

PCR reaction (in 50 µl)

34.5 µl	dH_2O
10.0 µl	5 x HF PCR reaction buffer
2.0 µl	5 mM dNTP
1.0 µl	Oligo 1
1.0 µl	Oligo 2
1.0 µl	FRT-PGK-gb2-neo-FRT PCR-template (tube 2)
0.5 µl	Phusion polymerase (5 U/µl)

- An annealing temperature of 57°- 62°C is optimal.
- PCR Profile: Initial denaturation step 30 sec 98°C; thirty cycles: 10 sec 98°C, 30 sec 55°C, 90 sec 72°C; final elongation step 10 min 72 °C.
- Check a 5 µl aliquot of the PCR product on a gel to ensure the PCR was successful. The size of the PCR product for the FRT-PGK-gb2-neo-FRT cassette is 1737 bp.
- Purify the PCR product either by running the whole PCR sample on an agarose gel and subsequent gel extraction or directly by Spin Column Purification (e.g. “Min Elute Gel Extraction Kit” or “; Qiagen). Adjust the DNA concentration to 100 ng/µl.

6.2 Transformation with Red/ET expression plasmid pRedET

Before starting with the experiment, please streak out the glycerol stock of the clone carrying you plasmid on LB plates conditioned with the appropriate antibiotics.

Day 1:

1. Set up an overnight culture. Pick one or two colonies and inoculate them in microfuge tubes containing 1.0 ml LB medium with appropriate antibiotics to select for your endogenous plasmid. Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

Day 2:

Before starting:

- Chill ddH_2O (or 10% glycerol) on ice for at least 2 h.
- Chill electroporation cuvettes (1 mm gap).
- Cool benchtop centrifuge to 2°C.

1. Set up a microfuge tube containing fresh 1.4 ml LB medium and inoculate with 30 µl of fresh overnight culture.

2. Culture for 2-3 h at 37°C, shaking at 1000 rpm.

3. Prepare the cells for electroporation
   Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (at 2°C). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Resuspend cells and keep the tube on ice.

4. Take the Red/ET Recombination protein expression plasmid pRedET (tube 1). Add 1 µl to your cell pellet. Mix briefly. Keep the tube on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

5. Electroporate at 1350 V, 10µF, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

6. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.

7. Incubate at 30°C for 70 min, shaking at 1000 rpm.
(The Red/ET expression plasmid pRedET will be lost at 37°C).

8. Using a small loop, plate 100 µl cells on LB agar plates containing tetracycline (3 µg/ml) plus the appropriate antibiotics for the plasmid. Use a loop to streak the control culture (tube 4: pSub-Hoxa11 + pRedET) on an LB agar plate with tetracycline (3 µg/ml) and ampicillin (100 µg/ml). Incubate the plates at 30°C overnight (or for at least 15 h). Protect the plates from light by wrapping them up, because tetracycline is sensitive to light. Make sure the cells stay at 30°C, otherwise the plasmid will be lost.

6.3 *Insertion of the FRT flanked PGK-gb2-neo cassette into a plasmid*

In the next step, prepare electro-competent cells from the plasmid hosts that contain the Red/ET expression plasmid, shortly after inducing the expression of the recombination proteins.
In advance, prepare the linear DNA fragment (the FRT-PGK-gb2-neo-FRT cassette) with homology arms that you will insert into your plasmid. Use tube 3 (FRT-PGK-gb2-neo-FRT PCR-product) and tube 4 (pSub-Hoxa11+pRedET) to perform a control experiment in parallel.

Day 3:

1. To start overnight cultures, pick one colony from the plate you obtained in 6.2, step 8 and inoculate one microfuge tube containing 1.0 ml LB medium plus Tetracycline (3 µg/ml) and the appropriate antibiotics for the plasmid [e.g. ampicillin (100 µg/ml) for the control]. Also pick one colony from the control plate. Puncture a hole in the lid of the tubes for air. Incubate the cultures while shaking at 30°C overnight.

Day 4:

Before starting:

- Chill ddH_2O (or 10% glycerol) on ice for at least 2 h.
- Chill electroporation cuvettes (1 mm gap).
- Cool benchtop centrifuge to 2°C.

2. The next day, set up 4 lid-punctured microfuge tubes (2 for your own experiment and 2 for control experiment) containing 1.4 ml each of fresh LB medium conditioned with the same antibiotics as in step 1. Inoculate two of them with 30 µl fresh overnight culture for your experiment, the other two with 30 µl of the overnight culture from the control experiment. Incubate the tubes at 30°C for 2 h shaking at 1100 rpm until OD600 ~ 0.3.

3. Add 50 µl 10% L-arabinose to half one of the tubes for your own experiment and to one of the control tubes, giving a final concentration of 0.3%-0.4%. This will induce the expression of the Red/ET Recombination proteins. Do not use D-arabinose. Leave the other tubes without induction as negative controls. Incubate all at 37°C, shaking for 45 min to 1 h.

   Note: It is important that cells are incubated at 37°C, the temperature at which all proteins necessary for the subsequent recombination are expressed. There are about 5 copies of this temperature-sensitive plasmid per cell, and during one hour there is approximately 1 doubling step, meaning any daughter cell will still have on average 2-3 copies left and will also go on expressing the recombination proteins. The plasmid is actually lost after electroporation and recombination, when cells are incubated at 37°C overnight.

4. Prepare the cells for electroporation
   Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (at 2°C). Discard the supernatant by quickly tipping it out twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH_2O (or 10% glycerol), pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Keep the tube on ice.

5. Add 1-2 µl (0.2-0.3 µg) of your prepared linear FRT-PGK-gb2-neo-FRT fragment with homology arms to each of the two microfuge tubes (induced and uninduced), and pipette the mixture into the chilled electroporation cuvette. In parallel, pipette 2 µl from tube 3 into each of the two tubes of the control.

6. Electroporate at 1350 V, 10 µF, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using an electroporation cuvette with a slit of 1 mm. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

7. Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37°C with shaking for 70 min. Recombination will now occur.

8. Streak the cultures with a loop (100 µl is sufficient, if necessary plate all) onto LB agar plates containing kanamycin (50 µg/ml) and the appropriate antibiotics for the plasmid [e.g. ampicillin (100 µg/ml) for the control]. The plates should not contain tetracycline; otherwise the Red/ET Recombination protein expression plasmid (pRedET) will either persist or the cells will die. Incubate the plates at 37°C overnight. The Red/ET Recombination protein expression plasmid (pRedET) will disappear at 37°C. You should obtain >100 colonies and the ratio of induced : uninduced bacterial colonies should exceed 10:1.

More than 95% of all colonies growing on the agar plates conditioned with the appropriate antibiotics will have successfully recombined copies of the plasmid. Please note that although most kanamycin-resistant colonies will contain the correct plasmid recombinant, in rare cases it is possible that secondary recombination, usually deletions between internal repeats in the plasmid, can also occur.

To confirm the correct recombination event, pick 10 – 20 colonies from your experiment and 2 from the control to isolate plasmid DNA. Also pick colonies from the original unmodified plasmid plates for DNA preparation and comparison. Perform mini-prep plasmid DNA isolation following the protocol of your choice and check these DNA preparations by restriction digestion.

A simple plasmid DNA isolation protocol is given below:

1. Spin down the 1.5 ml overnight cultures for 1 min at 13,000 rpm.
2. Discard the supernatant and resuspend the cell pellet in 200 µl buffer P1 with RNase (Qiagen).
3. Add 200 µl of buffer P2 (Qiagen) and mix by inverting the tube several times.
4. Add 200 µl of buffer P3 (Qiagen) and mix by inverting the tube several times. Leave the sample on ice for 10 min.
5. Spin down the white lysate at maximum speed for 10 min.
6. Transfer the clear supernatant into a new 1.5 ml-microfuge tube and add 0.50 ml of 2-propanol.
7. Mix by inverting the tube and spin down the DNA at maximum speed for 10 min.
8. Discard the supernatant and add 0.7 ml of 70% ethanol to rinse the pellet.
9. Spin down the DNA at maximum speed for 5 min and carefully discard the supernatant.
10. Dry the pellet under the speed vacuum for 2 min or leave the tube open on the bench for 5 to 10 min until the DNA pellet is completely dry. Do not overdry the pellet otherwise the DNA will become difficult to re-dissolve.
11. Carefully resuspend the dry DNA pellet in 50 µl ddH_20 or 10 mM Tris/HCl.

6.4 Verification of successfully modified plasmid by restriction analysis

Analyze an aliquot of your plasmid DNA by restriction digestion. For the control experiment, the restriction pattern for the original plasmid pSub-Hoxa11 after *Bgl*I digest is 422 bp, 692 bp, 1730 bp, 1836 bp, 1959 bp, 3485 bp and 7759 bp. The integration of the FRT-PGK-gb2-neo-FRT cassette leaves the smaller fragments intact but results in a cleavage of the 7759 bp fragment into two smaller ones with 4162 bp and 5234 bp respectively.

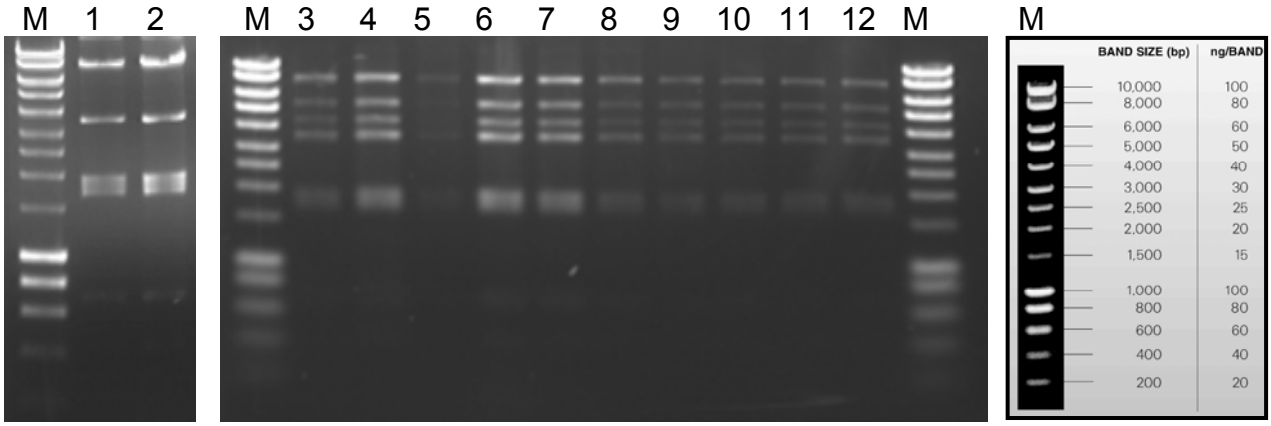


Figure 4: Restriction analysis of pSub-Hoxa11 (lanes 1-2) and 10 clones after insertion of the FRT-PGK-gb2-neo-FRT cassette (lanes 3-12) after *Bgl*I digestion. *M:* Hyperladder I (Bioline).

Although nearly all clones will show the expected restriction pattern for a successful integration of the FRT-PGK-gb2-neo-FRT cassette, the mother plasmid usually still persists in the cell. High copy plasmids like pBluescript or pSub11-Hoxa, which is used for the control experiment, replicate to up to several hundred copies per cell. Due to this high copy number, not all plasmid copies will be recombined at the same time resulting in a mixed “phenotype” where both plasmids are detectable side by side in the cell (see also Figure 4 for the control experiment).

To separate the modified plasmid from its unmodified mother plasmid, take a small amount of the isolated plasmid DNA from step 6.3 (about 1 ng) and re-transform it into a fresh aliquot of competent *E.coli* cells. Pick several colonies the next day, perform plasmid mini-prep plasmid DNA isolation following the protocol of your choice and check these DNA preparations by restriction digestion.

After the re-transformation the majority of the analyzed clones should show the restriction pattern for the modified plasmid only. For the control experiment, the restriction pattern for the plasmid pSub-Hoxa11-loxPneo after *Bgl*I digest is 422 bp, 692 bp, 1730 bp, 1836 bp, 1959 bp, 3485 bp, 4162 bp and 5234 bp (Figure 5).

Take one purified colony to perform step 6.5.

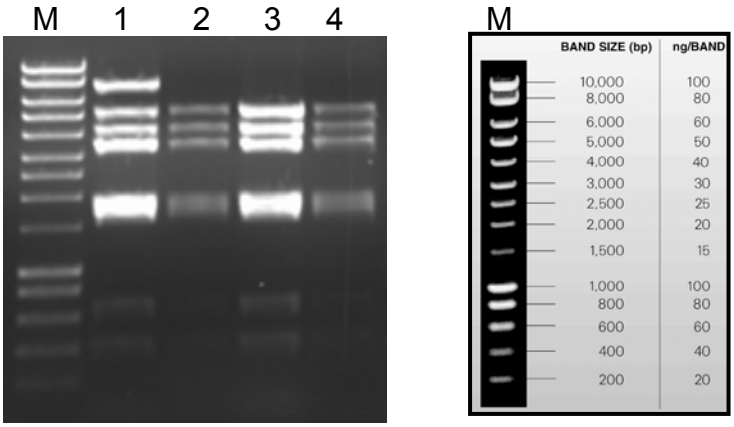


Figure 5: Restriction analysis (*Bgl*I digestion) of four colonies obtained in the control experiment after re-transformation. *M:* Hyperladder I (Bioline). While clone 1 still contains both plasmids in the cell as indicated by the 7759 bp fragment, all the three other clones show only the pattern expected for pSub-Hoxa11-FRTPneo.

6.5 Deletion of the kanamycin/neomycin selection marker by FLPe expression

In the next step, the kanamycin/neomycin selection marker will be removed by expression of FLPe recombinase. Prepare electro-competent cells from a clone harboring the plasmid with the FRT-flanked kanamycin/neomycin cassette and electroporate the plasmid pCI-FLPe, which expresses the recombinase. Take tube 6 to perform a control experiment in parallel. The plasmid pCI-FLPe has a pSC101 origin of replication which maintains low copy and replicates at 30°C. The plasmid will not propagate and will get lost when incubated at 37°C. The expression of FLPe-recombinase is driven by the thermo-sensitive promoter cI578 (λ_{PR} promoter). Therefore, the expression is repressed at 30°C and induced at 37°C. The plasmid carries a tetracycline resistance gene.

Day 1:

1. To start overnight cultures, pick one colony carrying the plasmid or 10µl from glycerol stock and inoculate one microfuge tube in 1.0 ml LB medium containing kanamycin (50 µg/ml) and the appropriate antibiotics for the plasmid [e.g. ampicillin (100 µg/ml) for the control]. Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

Day 2:

Before starting:

- Chill ddH_2O (or 10% glycerol) on ice for at least 2 h.
- Chill electroporation cuvettes (1 mm gap).
- Cool benchtop centrifuge to 2°C.

2. Set up a microfuge tube containing fresh 1.4 ml LB medium conditioned with the same antibiotics as in step 1 and inoculate with 30 µl of fresh overnight culture.

3. Culture for 2-3 h at 37 ºC, shaking at 1000 rpm.

4. Prepare the cells for electroporation
   Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (at 2°C). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH_2O (or 10% glycerol), pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Keep the tube on ice.

5. Add 1 µl of the expression plasmid pCI-FLPe (tube 5) to your cell pellet. Mix briefly and keep the tube on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

6. Electroporate at 1350 V, 10 µF, 600 Ohms. This setting applies to an Eppendorf® Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

7. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.

8. Incubate at 30°C for 70 min, shaking at 1000 rpm. (The expression plasmid pCI-FLPe will be lost at 37°C).

9. Using a small loop, plate 100 µl cells on LB agar plates containing tetracycline (3 µg/ml) plus kanamycin (50 µg/ml) and the appropriate antibiotics for the plasmid [e.g. ampicillin (100 µg/ml) for the control]. Incubate the plates at 30°C overnight (or for at least 15 h). Protect the plate from light by wrapping it up, because tetracycline is sensitive to light. Make sure the cells stay at 30°C, otherwise the plasmid will be lost.

10. Pick a single colony and grow the cells in 1 ml of LB medium plus the appropriate antibiotics for the targeting plasmid [ampicillin (100 µg/ml) for the control experiment] at 30°C for 2-3 h.

11. Change the temperature to 37°C and incubate overnight. FLPe protein will be expressed at this temperature and the FRT sites will be recombined. At the same time, the plasmid pCI-FLP cannot replicate any more and will get lost.

12. Prepare plasmid DNA and analyze the obtained clones by restriction digestion. About 60 – 80% of the flred fragments will be recombined. An additional re-transformation step is therefore necessary to remove the non-recombined plasmid.

6.6 *Verification of successfully modified plasmid by restriction analysis*

For the control experiment, the restriction pattern for pSub-Hoxa11-loxP after *Bgl*I digest is 422 bp, 692 bp, 1730 bp, 1836 bp, 1959 bp, 3485 bp and 7846 bp. Removal of the FRT-PGK-gb2-neo-FRT cassette results in the loss of two fragments (4162 bp and 5234 bp) and gain of one fragment (7846bp), see figure 6.

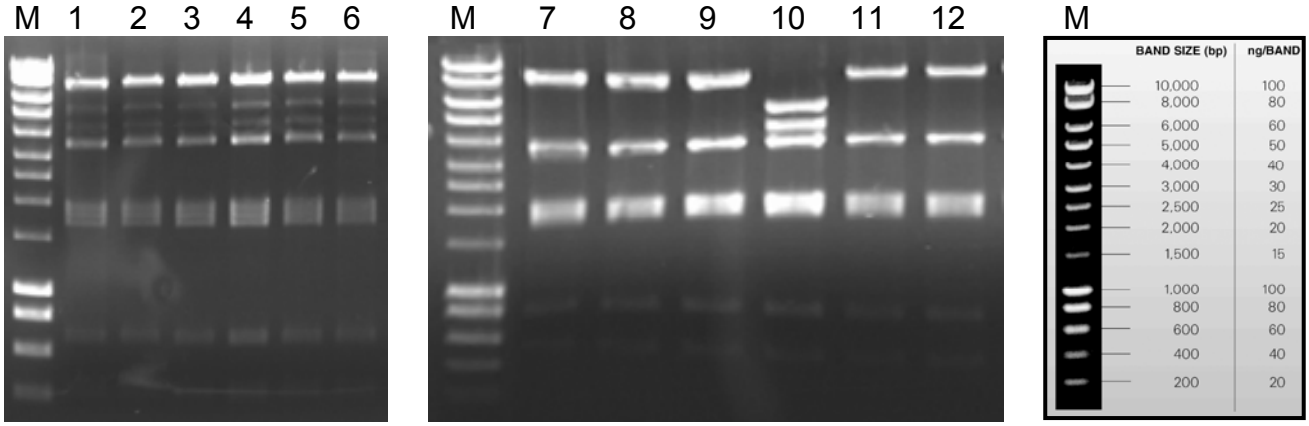


Figure 6: Restriction analysis of pSub-Hoxa11-FRT after *Bgl*I digest. Six colonies (lanes 1-6) were analyzed directly after the recombination step and six colonies after the re-transformation (lanes 7-12). *M:* Hyperladder I (Bioline). Directly after FLPe recombination nearly all clones analyzed still contain some copies of the pSub-Hoxa11-FRTneo plasmid as indicated by the presence of weak fragments at 4162 bp and 5234 bp (lanes 1 – 6). After the re-transformation step clones either contain the pSub-Hoxa11-FRT plasmid (lanes 7 – 9, 11 and 12) or the non-recombined pSub-Hoxa11-FRTneo (lane 10).

6.7 Maps and sequences

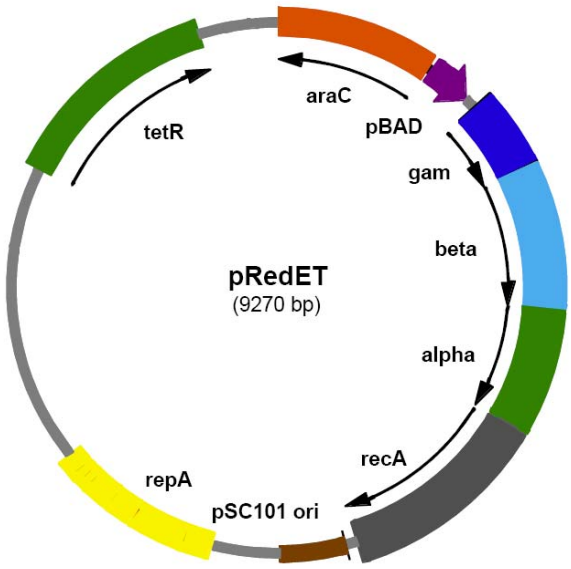


Figure 7: Map of the Red/ET expression plasmid pRedET. Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30°C. Expression of the Red/ET Recombination proteins is induced by L-arabinose activation of the pBAD promoter at 37°C.

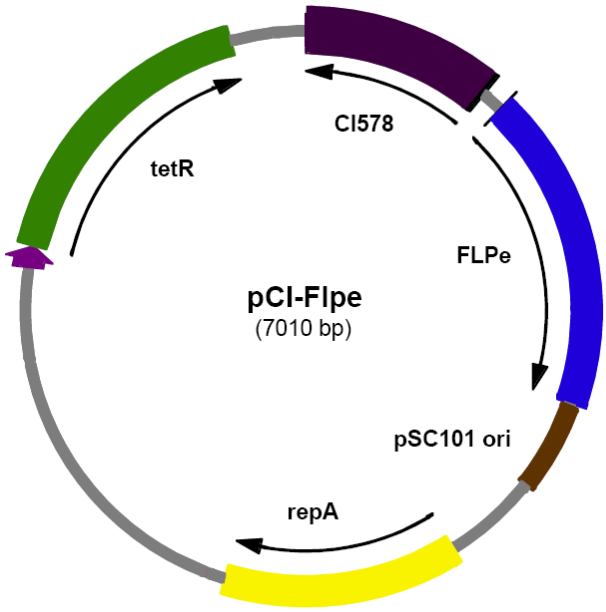
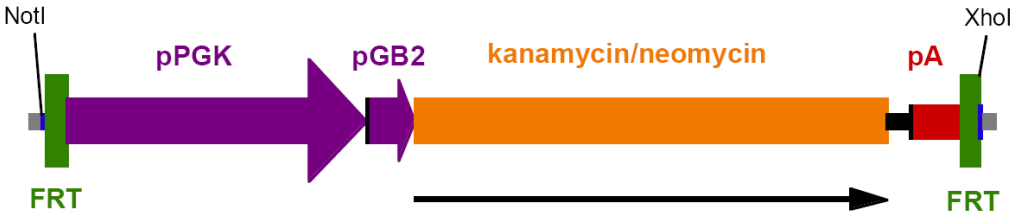


Figure 8: Map of the plasmid pCI-FLPe. Transformation of *E.coli* hosts with this plasmid is selected for by acquisition of tetracycline resistance at 30°C. Expression of the FLPe recombination proteins is induced by a temperature shift to 37°C.

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                                  NotI
   1 AATTAACCCTCACTAAAGG GCGGCCGC GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC ATTCTACCGG
  72 GTAGGGGAGG CGCTTTTCCC AAGGCAGTCT GGAGCATGCG CTTTAGCAGC CCCGCTGGGC ACTTGGCGCT
 142 ACACAAGTGG CCTCTGGCCT CGCACACATT CCACATCCAC CGGTAGGCGC CAACCGGCTC CGTTCTTTGG
 212 TGGCCCCTTC GCGCCACCTT CCACTCCTCC CCTAGTCAGG AAGTTCCCCC CCGCCCCGCA GCTCGCGTCG
 282 TGCAGGACGT GACAAATGGA AGTAGCACGT CTCACTAGTC TCGTGCAGAT GGACAGCACC GCTGAGCAAT
 352 GGAAGCGGGT AGGCCTTTGG GGCAGCGGCC AATAGCAGCT TTGCTCCTTC GCTTTCTGGG CTCAGAGGCT
 422 GGGAAGGGGT GGGTCCGGGG GCGGGCTCAG GGGCGGGCTC AGGGGCGGGG CGGGCGCCCG AAGGTCCTCC
 492 GGAGGCCCGG CATTCTGCAC GCTTCAAAAG CGCACGTCTG CCGCGCTGTT CTCCTCTTCC TCATCTCCGG
 562 GCCTTTCGAC C TGCAGC AGCACGTGTT GACAATTAAT CATCGGCATA GTATATCGGC ATAGTATAAT
 629 ACGACAAGGT GAGGAACTAA ACC ATG GGA TCG GCC ATT GAA CAA GAT GGA TTG CAC GCA GGT
                           1 Met Gly Ser Ala Ile Glu Gln Asp Gly Leu His Ala Gly
 691 TCT CCG GCC GCT TGG GTG GAG AGG CTA TTC GGC TAT GAC TGG GCA CAA CAG ACG ATC
  14 Ser Pro Ala Ala Trp Val Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile
 748 GGC TGC TCT GAT GCC GCC GTG TTC CGG CTG TCA GCG CAG GGG CGC CCG GTT CTT TTT
  33 Gly Cys Ser Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe
 805 GTC AAG ACC GAC CTG TCC GGT GCC CTG AAT GAA CTG CAG GAC GAG GCA GCG CGG CTA
  52 Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala Ala Arg Leu
 862 TCG TGG CTG GCC ACG ACG GGC GTT CCT TGC GCA GCT GTG CTC GAC GTT GTC ACT GAA
  71 Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val Leu Asp Val Val Thr Glu
 919 GCG GGA AGG GAC TGG CTG CTA TTG GGC GAA GTG CCG GGG CAG GAT CTC CTG TCA TCT
  90 Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu Val Pro Gly Gln Asp Leu Leu Ser Ser
 976 CAC CTT GCT CCT GCC GAG AAA GTA TCC ATC ATG GCT GAT GCA ATG CGG CGG CTG CAT
 109 His Leu Ala Pro Ala Glu Lys Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His
1033 ACG CTT GAT CCG GCT ACC TGC CCA TTC GAC CAC CAA GCG AAA CAT CGC ATC GAG CGA
 128 Thr Leu Asp Pro Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg
1090 GCA CGT ACT CGG ATG GAA GCC GGT CTT GTC GAT CAG GAT GAT CTG GAC GAA GAG CAT
 147 Ala Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu Glu His
1147 CAG GGG CTC GCG CCA GCC GAA CTG TTC GCC AGG CTC AAG GCG CGC ATG CCC GAC GGC
 166 Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala Arg Met Pro Asp Gly
1204 GAG GAT CTC GTC GTG ACC CAT GGC GAT GCC TGC TTG CCG AAT ATC ATG GTG GAA AAT
 185 Glu Asp Leu Val Val Thr His Gly Asp Ala Cys Leu Pro Asn Ile Met Val Glu Asn
1261 GGC CGC TTT TCT GGA TTC ATC GAC TGT GGC CGG CTG GGT GTG GCG GAC CGC TAT CAG
 204 Gly Arg Phe Ser Gly Phe Ile Asp Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln
1318 GAC ATA GCG TTG GCT ACC CGT GAT ATT GCT GAA GAG CTT GGC GGC GAA TGG GCT GAC
 223 Asp Ile Ala Leu Ala Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp
1375 CGC TTC CTC GTG CTT TAC GGT ATC GCC GCT CCC GAT TCG CAG CGC ATC GCC TTC TAT
 242 Arg Phe Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe Tyr
1432 CGC CTT CTT GAC GAG TTC TTC TGA GCGGGACTCTGGGGTTCGAATAAAGACCGACCAAGCGAC GTC
 261 Arg Leu Leu Asp Glu Phe Phe
1498 TGA GAGCTCCCTG GCGAATTCGG TACCAATAAA AGAGCTTTAT TTTCATGATC TGTGTGTTGG
                                                                      XhoI
1561 TTTTTGTGTG CGGCGCG GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC C TCGAGCCCTATAGTGAGTCGT
1634 ATTA
```

Figure 9: Map of the FRT-PGK-gb2-neo-FRT cassette.

7 Troubleshooting

Problems with the recombination reaction can be caused by a number of different factors. Please review the information below to troubleshoot your experiments.

We highly recommend performing a positive control experiment using the components provided in the kit.

For homologous recombination the two DNA molecules must share two regions of perfect sequence identity. Several wrong nucleotides in the homology region can completely abolish recombination. Since oligonucleotides are used to add the homology regions they have to be synthesized properly and be of excellent quality. Take into account that long oligonucleotides (especially if they are longer than 80bp) require additional purification steps, such as HPLC. Also note that the electronic sequences provided for BACs may not be 100% correct.

If you are trying to target a repeated sequence in your BAC or plasmid, you may experience problems because the homology region at the end of the linear fragment can go to more than one site. It is therefore best not to target repeats directly.

Observation:

<u>No colonies on your plate after Red/ET Recombination:</u>

If you do not obtain any colonies after recombination, the following parameters should be checked:

1) The PCR product

- could be wrong (check it by restriction digest or sequencing)
- could be degraded (check an aliquot on an agarose gel)
- could have incorrect homology arms. Please double-check the oligonucleotides used to generate the PCR product for quality and correctness. If necessary verify the sequence by sequencing the PCR product.
- may not be enough; increase the amount of PCR product from approximately 100 ng to up to 500 ng. Please take into consideration that you may also increase non-unspecific background.

2) The plasmid or BAC

- may be instable and may have rearranged. Digest the BAC and run on a gel (preferably PFGE) to confirm the approximate size.
- may contain some repeats in the region you are targeting. Re-check sequence.
- could be wrong; make sure that you have the right plasmid/BAC by isolating DNA and checking the region of the homology arms by PCR and/or sequencing. If necessary sequence the PCR product to verify the region of homology. Some BACs are wrongly annotated, inherently instable or a mixture of more than one BAC.

3) The Red/ET reaction did not take place because

- there was no expression plasmid present in the cells; e.g. the cells were grown at 37°C instead of 30°C (check for tet resistance).
- no or the wrong type of arabinose was used for induction (please make sure you use L-arabinose!)
- some strains (e.g. JM109, DH5alpha) are less efficient in Red/ET Recombination than others. DH10B, HS996, GeneHogs or TOP10 are our preferred strains.
- in very rare cases an elongation of the reaction time for the recombination from 70 min (incubation of electroporation) to up to four hours is necessary for successful recombination.

4) Problems with and after the electroporation:

- cells are not competent enough to take up the linear DNA fragment. Please make sure that the cells were kept on ice and that the water (or 10% glycerol) is sufficiently cold. Linear DNA has been shown to be about 10^4-fold less active than DNA transformed in circular form (Eppendorf Operation Manual Electroporator 2510 version 1.0). Make sure that the time constant is around 5 ms.
- please make sure that there is no arching during the electroporation process.
- please make sure that after electroporation the cells are plated on the appropriate concentration of antibiotics depending on the copy number of the plasmid or BAC.

Similar number of colonies on both plates, the induced and the uninduced one:

If you obtain a **high number** of colonies on both plates, it indicates that there are still traces of the circular (or supercoiled) plasmid used for preparing the linear fragment left in the sample. Since the transformation efficiency of linear fragments is 10^4-fold less than that of circular DNA molecules you may obtain a background even if no traces were visible on an agarose gel.
If the linear DNA fragment was obtained by restriction digestion, use less DNA and gel purify the fragment! If the linear cassette was obtained by PCR, set up a *Dpn*I digest for your PCR product and gel purify it at the end!

If you obtain a very **low number** of colonies on both plates, it indicates that the overall efficiency of Red/ET Recombination is very low. In this case please check all parameters mentioned in the section entitled: “no colonies after Red/ET Recombination”.

You cannot separate the recombined plasmid from the non-recombined one after recombination even after re-transformation (high copy plasmid!):

In very rare cases we have observed that after recombination it is difficult to separate the original plasmid from the recombined one. If you cannot separate them by dilution of the plasmid and re-transformation, you can choose a single cutting restriction enzyme and digest the plasmid for a few minutes. After re-transformation the two plasmids should be separated even when they were tangled before.

8 References and Patents

8.1 References

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8.2 Patents

Red/ET recombination is covered by one or several of the following patents and patent applications:

- Stewart, A.F., Zhang, Y., and Buchholz, F. 1998. Novel DNA cloning method. *European Patent No.1034260 (issued on 12th of March, 2003), United States Patent No 6,509,156.*

- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 1999. Methods and compositions for directed cloning and subcloning using homologous recombination. *United States Patent No. 6,355,412 (issued on 12th of March, 2002).*

- Youming Zhang, A. Francis Stewart, and Joep P.P. Muijrers. 2001. Improved RecT or RecET cloning and subcloning method. *European Patent Application No. 01 117 529.6*

- Stewart, A.F., Zhang, Y., and Muyrers, J.P.P. 2001. Recombination method. European Patent Application No. 0103276.2

These patents and patent applications are owned by Gene Bridges, or owned by the EMBL and exclusively licensed to Gene Bridges.

9 Purchaser Notification/Warranty

This product is the subject of European Patent No.1034260 (issued on 12.3.2003) (or PCT/EP98/07945) and United States Patent No. 6,355,412 (issued on 12th of March, 2002). The purchase of this product conveys to the purchaser the non-transferable right to use this product for research purposes only. The purchaser can not sell or otherwise transfer this product or its components to a third party. No rights are conveyed to the purchaser to use this product or its components for a commercial purpose. Commercial purposes shall include any activity for which a party receives consideration of any kind. These may include, but are not limited to, use of the product or its components in manufacturing, to provide a service, information or data, use of the product for diagnostic purposes, or re-sale of the product or its components for any purpose, commercial or otherwise.

The use of homologous recombination for commercial purposes may infringe the intellectual property covered by the EP 419,621 patent family.

Products containing the araB promoter are sold under patent license for **research purposes only** and are non-transferable. Inquiries for any commercial use, including production of material to be sold commercially or used in production or in product development efforts which includes efforts toward regulatory approval, should be made directly to Xoma Corporation, Berkeley, California.

Xoma Corporation
2910 Seventh Street
Berkeley, CA 94710

Limited Warranty
Gene Bridges is committed to providing customers with high-quality goods and services. Gene Bridges assumes no responsibility or liability for any special, indirect, incidental or consequential loss or damage whatsoever. This warranty limits Gene Bridges GmbH's liability only to the cost of the product.

10 Other products available from Gene Bridges

General information

- Kits are available for non-commercial research organizations. Commercial companies or for-profit organizations require a sub-license to use Red/ET Recombination.

The complete product list as well as all information about how to order kits in your country is given on our website: www.genebridges.com

K001: Quick and Easy BAC Modification Kit

Description:

- This kit is designed to modify any type of bacterial artificial chromosomes (BACs) within 1-2 weeks by using a kanamycin/ neomycin cassette
- This kit is optimized for basic modifications such as insertions or deletions of fragments in any type of bacterial artificial chromosomes (BACs) leaving a selectable marker gene.
- This kit can also be used to work on bacterial chromosomes and common ColE1 origin plasmids.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- BAC host *E.coli* strain HS996 already carrying the Red/ET plasmid.
- Tn5-neomycin resistance template to be used for your own experiments.
- Positive controls to introduce a Tn5-neo cassette in a 150 kb BAC.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K002: Counter-Selection BAC Modification Kit

Description:

- This kit is designed to modify any type of bacterial artificial chromosomes (BACs) within 2-3 weeks by using a counter-selection cassette
- The kit is designed for advanced BAC modification such as introducing short sequences (e.g. point mutations, loxP sites, restriction sites, etc.), insertion or deletion of non-selectable marker genes, fragment exchange without leaving a selection marker or any unwanted sequences.
- The included counter-selection cassette pRpsL-neo is based on streptomycin selection which shows a much higher efficiency than pSacB-neo or comparable systems.
- This kit can also be used to work on bacterial chromosomes and common ColE1 origin plasmids.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- BAC host *E.coli* strain HS996 already carrying the Red/ET plasmid.
- pRpsL-neomycin template to be used for your own experiments.
- Positive controls to introduce a point-mutation in a 150 kb BAC.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K003: BAC Subcloning Kit

Description:

- This kit is optimized for subcloning of DNA fragments from BACs and cosmids.
- No restriction sites necessary.
- Fragments up to 20 kb can easily be subcloned in one step.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- Linear vector carrying a ColE1 origin of replication plus ampicillin resistance gene to be used for the subcloning experiment.
- Positive controls to subclone a 15 kb fragment from a control BAC into the vector delivered with the kit.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K004: Quick and Easy Conditional Knockout Kit (FRT/FLPe) and K005: Quick and Easy Conditional Knockout Kit (loxP/Cre)

Description:

- This kit is designed to integrate FRT or loxP sites into large vectors at any position within 2 weeks.
- Single FRT or loxP sites are inserted by Red/ET recombination of FRT or loxP flanked functional cassettes into any designated locus with subsequent removal of the selection marker by FLPe or Cre recombinases.
- Conditional targeting constructs can be generated either by a repetitive insertion of the functional cassette supplied with the kit or by combination with other functional cassettes offered by Gene Bridges.
- The functional cassette supplied with the kit (FRT-PGK-gb2-neo-FRT or loxP-PGK-gb2-neo-loxP) combines a prokaryotic promoter (gb2) for expression of kanamycin resistance in *E. coli* with an eukaryotic promoter (PGK) for expression of neomycin resistance in mammalian cells.
- High Red/ET efficiency plus convenient removal of the Red/ET Recombination protein expression plasmid pRedET after recombination.

Contents:

- Red/ET Recombination protein expression plasmid pRedET. Any *E. coli* strain can be made Red/ET proficient by transformation with this plasmid.
- FRT or loxP flanked kanamycin/neomycin resistance template (FRT-PGK-gb2-neo-FRT or loxP-PGK-gb2-neo-loxP) to be used for your own experiments.
- Expression plasmid for FLPe or Cre site specific recombinase in *E. coli* cells.
- Positive controls to introduce a single FRT or loxP site into a 15 kb high copy plasmid.
- Detailed protocols, descriptions of plasmids, maps and sequences.

K006: Quick and Easy *E.coli* Gene Deletion Kit

Description:

- This kit is designed to knock-out or alter genes on the *E. coli* chromosome in less than one week.
- Red/ET recombination allows the exchange of genetic information in a base pair precise, specific, and reliable manner.
- A FRT-flanked kanamycin resistance marker cassette is supplied with the kit and can be used to replace a gene on the *E. coli* chromosome.
- Red/ET recombination can replace fragments as large as 30kb from the chromosome.
- The use of a FRT-flanked resistance cassette for the replacement of the targeted gene allows the subsequent removal of the selection marker by a FLP-recombinase step, if required. (FLP expression plasmids can be purchased from Gene Bridges).
- Multiple knock-outs can be generated either by repetitive insertion of the functional cassette supplied with the kit or by combination with other functional cassettes offered by Gene Bridges.
- Strictly controlled recombination process due to an optimized design of the pRedET expression plasmid. The genes for the recombination proteins are under the control of an inducible promoter and the plasmid carries a temperature-sensitive origin of replication for the convenient removal of the plasmid after recombination.

Contents:

- Two Red/ET Recombination protein expression plasmids pRedET (tet) and pRedET (amp). Any *E. coli* strain can be made Red/ET proficient by transformation with these plasmids.
- FRT flanked kanamycin resistance template (FRT-PGK-gb2-neo-FRT) to be used for your own experiments.
- Positive controls to replace the gene for the mannose transporter (man*X*) on the *E. coli* chromosome.
- Detailed protocols, descriptions of plasmids, maps and sequences.

Additional functional cassettes:

- A001: Pro- and Eukaryotic Neomycin Selection Cassette (PGK-gb2-neo)
- A002: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette (FRT-PGK-gb2-neo-FRT)
- A003: loxP flanked, Pro- and Eukaryotic Neomycin Selection Cassette (loxP-PGK-gb2-neo-loxP)
- A004: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette plus loxP site (FRT-PGK-gb2-neo-FRT-loxP)
- A005: FRT flanked, Pro- and Eukaryotic Neomycin Selection Cassette plus loxP site 2nd version (loxP-FRT-PGK-gb2-neo-FRT)
- A006: FRT flanked Chloramphenicol Selection Cassette (FRT-cm-FRT)
- A007: loxP flanked Chloramphenicol Selection Cassette (loxP-cm-loxP)
- A008: FRT flanked Ampicillin Selection Cassette (FRT-amp-FRT)
- A009: loxP flanked Ampicillin Selection Cassette (loxP-amp-loxP)
- A010: FRT flanked, Pro- and Eukaryotic Hygromycin Selection Cassette (FRT-PGK-gb2-hygro-FRT)
- A011: loxP flanked, Pro- and Eukaryotic Hygromycin Selection Cassette (loxP-PGK-gb2-hygro-loxP)

Additional strains and plasmids:

- A104: Enhanced FLP Expression Plasmid 707-FLPe with tetracycline resistance marker for use in *E. coli* only
- A105: Enhanced FLP Expression Plasmid 708-FLPe with chloramphenicol resistance marker for use in *E. coli* only
- A112: Cre Expression Plasmid: 705-Cre (cm resistance marker)
- A113: Cre Expression Plasmid: 706-Cre (tet resistance marker)
- A201: Enhanced Eukaryotic FLP Expression Plasmid: pCAGGS-FLPe

11 **DNA Engineering Services Available from Gene Bridges**

Instead of performing your own DNA manipulations, let the Gene Bridges DNA Engineering Service Team do the work for you. We work for many commercial and research organizations across the world to provide DNA modifications in low- or high-copy plasmids, cosmids, BACs and the *E.coli* chromosome.

The available DNA modifications are:

- Insertion of a selectable or non-selectable marker cassette
- Deletion of sequences of any size, ranging from 1 bp up to more than 100 kb with or without leaving a selectable marker
- Replacement of genes on the *E.coli* chromosome
- Point mutations
- Fusions
- Introduction of site specific targeting sites (loxP, FRT, etc.)
- Insertion of restriction enzyme recognition sites
- Subcloning of DNA sequences up to 60 kb
- Transferring DNA fragments into multiple destination vectors
- BAC and cosmid stitching
- Substitutions

Contact our DNA Engineering Service by email at contact@genebridges.com,
or go to www.genebridges.com for details and prices.

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